Assessment of ionic quenching on soil ATP bioluminescence reaction

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Abstract

Co-extracted ions with ATP from soils may interfere with ATP luciferin–luciferase luminescence reaction when ATP is assayed. The effects were investigated in a typical concentration range of cations and anions potentially extractable in soils. A commercial ATP assay product (Sigma Chemical Co.) was used. Significant quenching is evidenced from a concentration of 0.10 mM with Cu 2+ and Zn 2+, and 1.00 mM with Ca 2+. The order of quenching at 1.00 mM was: Cu 2+ > Zn 2+ > Ca 2+ = Na + = Mg 2+, while Mg 2+ = Mn 2+, both Ca 2+ and Na + > Mn 2+. The quenching was found to be much more severe with selected special heavy metal cations with quenching in the order: Ti 3+ > Hg 2+ > Cr 3+. Because cation quenching can be alleviated by addition of EDTA, three forms of EDTA (Mg, Na and acid EDTA) were tested for their suitability for the assay. The Mg-EDTA was found superior to the other two. Presence of PO 4 3− at concentrations of 0.01 and 0.05 mM, and NO 3− at 0.01 and 0.10 mM, significantly enhanced ATP light emission (8–13%). However, SO 4 2− at similar concentrations significantly decreased light emission. The quenching by CO 3 2− and Cl− was only observed at high concentrations (3.20 mM and up). The order of quenching for the anions at a concentration of 6.4 mM was: PO 4 3− > CO 3 2− > SO 4 2− > NO 3− > Cl−. Enhanced or depressed light emission induced by ions would produce significant over or underestimation of soil ATP. While addition of Mg-EDTA may alleviate cation quenching, the interference from anions may require the ATP assay standards be prepared in a solution of similar chemical composition to that in soil ATP extracts. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: ATP; Soil extracts; Luciferin–luciferase; Cations; Anions

1. Introduction

The bioluminescence reaction of adenosine triphosphate (ATP) with firefly luciferin–luciferase was first recognized by McElroy (1947). Light emission from the firefly lantern extract could be induced by the addition of ATP and the emission is proportional to the amount of ATP added. Soon after the principle was discovered, the bioluminescence reaction became a popular sensitive technique for measuring ATP in physiological research. During the last two decades, the luciferin–luciferase system has been used increasingly for ecological and environmental studies.

Initial extractants used in soil ATP studies were developed from methods used to measure ATP in pure cell cultures. However, when these extractants were applied to soil samples, there were severe shortcomings. The ATP concentration is very low in soils and factors such as incomplete cell lysis and ATP extraction, ATP adsorption by soil organic and inorganic substances and hydrolysis during the extraction process may significantly lower the efficiency of the soil ATP measurement (Webster et al., 1984). Reagents preventing ATP decomposition during extraction would inhibit ATP bioluminescence reaction. It is unlikely that a single extractant would be capable of inactivating ATPase enzymes in microbial cells during extraction, and not affect the bioluminescence reaction in the assay. Often, to overcome these difficulties, the components of soil ATP extractants are quite complicated and contain high concentrations of strong acid (Lee et al., 1971; Greaves et al., 1973; Karl and LaRock, 1975; Jenkinson and Oades, 1979; Christensen and Devol, 1980; Webster et al., 1984).

Cations and anions are abundant in most soils. Extraction of cations and anions along with soil ATP is unavoidable. The presence of ions in the extraction solution may affect the efficiency of the light emission by altering the activity of luciferin–luciferase. Aleldor et al. (1966) reported that cation and anion inhibition occurred in the following orders

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when testing ATP in red blood cells:

Cations: $\text{Ca}^{2+} > \text{K}^+ > \text{Na}^+ > \text{Rb}^+ > \text{Li}^+ > \text{choline} ;$

Anions: $\text{I}^- > \text{H}_2\text{PO}_4^- > \text{Br}^- > \text{ClO}_4^- > \text{Cl}^- > \text{F}^- > \text{CH}_3\text{COO}^- .$

Karl and LaRock (1975) observed the following order of inhibition in testing ATP in marine sediments:

Cations: $\text{Ca}^{2+} > \text{K}^+ > \text{Na}^+ > \text{Mg}^{2+} ;$

Anions: $\text{CO}_3^{2-} > \text{PO}_4^{3-} > \text{SO}_4^{2-} > \text{Cl}^- .$

The quenching order determined by Aledort et al. (1966) was at a concentration of 97 mM for all the cations and anions. Karl and LaRock (1975) used a series of ion concentrations from 1 to 100 mM in the final reaction mixture with no measurements made for 0 to 1 and 1 to 25 mM ranges. Afghan et al. (1977) investigated the quenching effect of Mn$^{2+}$ starting from the lowest concentration of 0.25 mM. While these studies have presented useful information, the concentrations of ions tested were very high and are unlikely to occur in ATP extracts from soils. Only a limited number of elements relevant to soils was tested.

For a given molar concentration of a cation, the light emission efficiency decreases drastically with lowered ATP concentration. The inhibition of light emission is more pronounced at lower ATP levels (Karl and LaRock, 1975). The ionic inhibition in the luciferin–luciferase reaction is not only a function of the nature of the cation or anion and its concentration, but also the concentration of ATP in the reaction mixture. The ATP concentration in soils is very low compared with animal or plant tissue because minerals often constitute more than 95% of the soil weight and only a very tiny fraction of the soil organic components is living organisms. The ionic quenching effects in solutions at the concentrations typically found in soil extracts, the concentrations in the final reaction mixture were designed to be 0.000 (control), 0.001, 0.005, 0.010, 0.100 and 1.000 mM, and 0.00 (control), 0.01, 0.05, 0.10, 0.50, 1.00, 3.20 and 6.40 mM for the cations and anions, respectively.

The concentration of luciferin also affects the rate of bioluminescence reaction as it participates directly in the reaction. In addition to ATP, the enzyme-luciferase uses luciferin and oxygen as substrates. The rate limiting step for the overall reaction was the formation of the luciferin–adenylate complex and Mg-ATP functions as a cofactor. The final product is oxy-luciferin (Karl and Holm-Hansen, 1976). Only when all of the reactants are in excess, light emission is directly proportional to the concentration of ATP in the reaction mixture (De Luca and McElroy, 1978). The light can be measured from either the initial rise of the luminescent curve, the peak height, or some integrated portion of the subsequent decay curve over a known period.

The objective of this experiment was to investigate the influence of ions in concentrations typically found in soil ATP extracts on the bioluminescence reactions.

2. Materials and methods

2.1. Ion selection and concentration

The following cations commonly present in soils were selected: $\text{Cu}^{2+}, \text{Zn}^{2+}, \text{Ca}^{2+}, \text{Mg}^{2+}, \text{Mn}^{2+}$ and $\text{Na}^+$. The specific heavy metal cations: $\text{Ti}^{3+}, \text{Hg}^{2+}$ and $\text{Cr}^{3+}$ that could pollute soil through industrial activity were also chosen. Because $\text{Cl}^-$ often has the least quenching effect on the ATP firefly luciferin–luciferase reaction (Denburg and McElroy, 1970; Karl and LaRock, 1975) and chloride salts are easy to obtain and prepare, chloride salts were used. The following anions that commonly exist in soils were tested: $\text{PO}_4^{3-}, \text{CO}_3^{2-}, \text{SO}_4^{2-}, \text{NO}_3^-$ and $\text{Cl}^-$. Because $\text{Na}^+$ usually exhibits minimal quenching effects (Aledort et al., 1966; Karl and LaRock, 1975), the sodium salt forms were used.

Solutions containing $\text{Cu}^{2+}, \text{Zn}^{2+}, \text{Ca}^{2+}, \text{Na}^+, \text{Mg}^{2+}, \text{Mn}^{2+}$ and $\text{Hg}^{2+}$ were prepared using Certified Atomic Absorption Standard Reference Solution (1000 mg L$^{-1}$, Fisher Scientific Company) through multiple dilution. Distilled-deionized water was used to dilute the reference solution and was used throughout the experiment.

The $\text{Ti}^{3+}$ and $\text{Cr}^{3+}$ working solution was prepared as follows:

1. $\text{Ti}^{3+}$ of 5.0 mM was prepared using the standard reference solution (1000 mg L$^{-1}$ Solution: Titanium Metal. Solvent: Dilute Hydrochloric Acid. Fisher Scientific Company). A 50 ml aliquot of the reference solution was stirred with gentle heating in a beaker under vacuum for about 30 min to remove $\text{HCl}$. The solution was then decanted into a 50 ml volumetric flask and made up to the volume. A 23.94 ml aliquot was transferred into another 50 ml volumetric flask and made up to volume. The final solution was brown color with a pH of 2.

2. $\text{Cr}^{3+}$ of 10 mM solution was prepared by dissolving a 0.0026 g of chromium metal dust (100 mesh, Matheson Coleman & Bell Manufacturing Chemicals) in 2 ml of 0.1 M $\text{HCl}$ and about 20 ml of water. The solution was gently heated for 30 min. to complete the reaction, then further heated and stirred under vacuum (water pump) for 30 min. to remove $\text{HCl}$. The solution was made up to a final volume of 50 ml with water.

Soil ATP is often extracted with a 1:10 soil: extractant ratio. The extracts may be diluted or neutralized before assay. To better coincide with the concentrations typically present in soil extracts, the concentrations in the final reaction mixture were designed to be 0.000 (control), 0.001, 0.005, 0.010, 0.100 and 1.000 mM, and 0.00 (control), 0.01, 0.05, 0.10, 0.50, 1.00, 3.20 and 6.40 mM for the cations and anions, respectively.

2.2. Preparation of ATP standard, enzyme and buffer

The ATP Assay Kit from Sigma Chemical Company
(Stock No. FL-AAS) was selected. To prepare the ATP standard, 0.950 mg of ATP from the kit was dissolved in 10 ml of water. A 0.500 ml aliquot of the solution was made up to 50 ml volume with cool water. A 0.2 ml aliquot of diluted solution was placed in a plastic vial and 9.8 ml of HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate, pH 7.75, 25 mM) was added. The standard (19 ng ATP ml⁻¹) was thoroughly mixed, then kept on ice.

The enzyme solution was prepared by first dissolving the ATP Assay Mix (Stock no. FL-AAM) in 5.00 ml of water and the Dilution Buffer (Stock No. FL-AAB) in 50 ml of water. The ATP Assay Mix (5 ml) was thoroughly mixed with 20 ml of the buffer.

The advantage of using purified commercial products for routine soil ATP assay is apparent. Commercial ATP Assay Mix products are easy to obtain and convenient to prepare. The products contain purified luciferase along with sufficient luciferin. Crude luciferase preparations may contain many other contaminating enzymes and some of these are capable of producing ATP from other sources, thus resulted in overestimation of ATP (Karl, 1978; Tate and Jenkinson, 1982). As well, the kit often provides other chemicals such as ethylene diamine tetraacetic acid (EDTA) and buffers. These reagents are necessary to stabilize the enzyme, maintain the solution pH in an optimum range for the reaction, reduce quenching, and enhance the light emission (Aledort et al., 1966; Webster and Leach, 1980). Using commercial products may pave the road towards standardization of soil ATP assay in laboratories.

2.3. Optimizing pH for the luminescence reaction

There is a pH sensitive anion binding site on luciferase which influences both the enzymatic activity and the wave length of light emitted. pH as high as 9 or as low as 5 would completely quench the reaction (Tobin et al., 1978). Most studies have used a pH of 7 to 8 (Van Dyke et al., 1969; Stanley and Williams, 1969; Kimmich et al., 1975). Webster et al. (1980) recommended a pH of 7.8, which is used by commercial products (Mono Research Laboratories, 1981; Sigma, 1987). A pre-test found that a 3:1 ratio (volume basis) of the buffer, enzymes and other reagents from the ATP Assay Mix to the salt solution was sufficient to produce a pH of 7–8 in the final mixture solution. The actual ratio used was 4:1.

2.4. The assay procedure

Light emission from the ATP luminescence reaction was measured using a Lumac Model 1070 ATP luminometer (Lumac Systems Inc., P.O. Box 2805, Titusville, FL 32780, USA).

The reagents were dispensed to a cuvette using a mini-volume multi-tip precision pipette:

1. Inject 300 µl of water for the control (without a cation or an anion solution) and 250 µl for the treatments;
2. Inject 50 µl of the HEPES buffer;
3. Inject 50 µl of the diluted cation or anion solution to treatment cuvette;
4. Inject 50 µl of the ATP standard. No ATP was added to the cuvette for background measurement; and
5. Place the cuvette into the luminometer and inject 100 µl enzyme solution. The instrument setting was a 5 second delay after injection followed by a 10 s integrated count.

Four replicates of each assay were performed.

2.5. Statistical analysis

The data were analyzed using SAS program (SAS Inst., 1989). A paired t test was used to compare the difference between the control and the treatment at different concentrations. An ANOVA was employed and protected LSDs (P < 0.05) were used to determine quenching order at the specific concentrations.

3. Results

3.1. Cation

In comparison with the control, no apparent quenching was observed for any cations at concentrations lower than 0.01 mM (Fig. 1). However, Cu²⁺ and Zn²⁺ began to show significant quenching effects at a concentration of 0.1 mM, followed by Ca²⁺ and Na⁺ at 1 mM. No significant quenching was observed for Mg²⁺ and Mn²⁺ at the highest
concentration (1 mM). The greatest depression of light emission was 52.9% with addition of Cu\(^{2+}\) at a concentration of 1 mM. As accurate as a 0.5% of coefficient of variation (CV) may be obtainable in pure ATP solution assay (Mono Research Laboratories, 1981). Most researchers have obtained a CV less than 10%, which often includes errors from soil sampling, extraction diversity and assay. The standard error (SE) was calculated as a percentage of measurement means in our study. Except for the largest SE (\(\pm 11.8\%\)) produced with Cu\(^{2+}\) at a concentration of 1.0 mM, the SEs were within \(\pm 5\%\) of the mean value for the significant differences presented in Fig. 1. At a concentration of 1 mM, the order of the quenching effect was as follows:

\[
\text{Cu}^{2+} > \text{Zn}^{2+} > \text{Cu}^{2+} = \text{Na}^+ = \text{Mg}^{2+}, \text{while Mg}^{2+} = \text{Mn}^{2+}, \text{both Ca}^{2+} \text{and Na}^+ > \text{Mn}^{2+}.
\]

3.2. Specific heavy metal cation

Addition of the specific heavy metal cations, Hg\(^{2+}\), Cr\(^{3+}\) and especially Ti\(^{3+}\) significantly reduced the light emitted from the ATP bioluminescence reaction (Fig. 2). At a concentration of 0.005 mM, Ti\(^{3+}\) exhibited significant quenching effect and light emission ceased at a concentration of 0.1 mM. The quenching effect for Hg\(^{2+}\) occurred at a higher concentration than did Ti\(^{3+}\). Cr\(^{3+}\) had the lowest quenching effect. The order of quenching was: Ti\(^{3+}\) > Hg\(^{2+}\) > Cr\(^{3+}\).

The SE value for Ti\(^{3+}\) was \(\pm 2.6, \pm 6.1\) and \(\pm 35.4\%\) with concentration of 0.005, 0.010 and 0.100 mM, respectively. At a concentration of 1.000 mM, the SE was \(\pm 32.8\) and \(\pm 68.7\%\) with Hg\(^{2+}\) and Cr\(^{3+}\), respectively. These SEs increased with increased concentration and were much larger than those presented in Fig. 1.

3.3. Added EDTA

Addition of different forms of EDTA produced different effects on ATP light emission (Fig. 3). While no significant quenching was observed with addition of acid-EDTA up to a concentration of 0.2 mM [EDTA acid is rather insoluble in water (about 0.02 g/100 ml water at 22°C) (Wu Han University, 1979)], addition of Na-EDTA increased light emission at the concentrations of 0.1 and 0.2 mM by 7.4 and 14.5%, respectively. Further increases in the concentration of acid-EDTA or Na-EDTA tended to decrease light emission. Addition of Mg-EDTA did not interfere with light emission up to concentrations of 1.00 mM. The quenching order for depression of light emission is: acid-EDTA > Na-EDTA > Mg-EDTA.

3.4. Anion

While enhanced ATP light emission was observed for PO\(_4^{3-}\) at concentrations of 0.01, 0.05 and 0.10 mM and for NO\(_3^-\) at 0.1 mM, the increases were in the range of 8.1–13.2%, at similar concentrations SO\(_4^{2-}\) significantly decreased light emission (Fig. 4). For CO\(_3^{2-}\) and Cl\(^-\), significant quenching was observed only at high concentrations.
The greatest quenching observed with Cu\(^{2+}\) was contrary to the results of Tobin et al. (1978). In seven cations tested (Cu\(^{2+}\), Mg\(^{2+}\), Al\(^{3+}\), Fe\(^{3+}\), Fe\(^{2+}\), Pb\(^{2+}\) and Mn\(^{2+}\)), they reported that Cu\(^{2+}\) showed the second greater quenching effect after Mn\(^{2+}\) and although the quenching of Mn\(^{2+}\) was reduced with addition of EDTA, the apparent recovery of spiked ATP was still lower than for other metals. There is a recent trend toward the use of organic wastes such as sewage sludge, as low quality fertilizers for agricultural systems (Page et al., 1987; Liao and Christie, 1998). Sewage sludge can be rich in metals. A survey of sludge composition conducted by Sommers (1977) revealed that the mean concentration of Cu was 1210 mg kg\(^{-1}\) with the maximum of 10,400 mg kg\(^{-1}\) in 208 samples collected in the USA. The mean concentration of Zn was 2790 mg kg\(^{-1}\) with a maximum of 27,800 mg kg\(^{-1}\). Copper, especially Cu applied in organic wastes is not often easily leachable but remains in the topsoil (Chang et al., 1991; Barbarick et al., 1998). Continued application of high metal sludge at high rates will result in a high concentration of Cu and Zn in top soils. The strong acids often used in soil ATP extractions to lyse organisms and prevent ATP decomposition and adsorption to clay are H\(_2\)SO\(_4\), HClO\(_4\) and H\(_3\)PO\(_4\). These acid extracts have a much higher ability to extract Cu\(^{2+}\) and Zn\(^{2+}\) from the soil than neutral or alkaline extractant (Parkinson and Paul, 1982). Although neutralization of ATP extractants is often required before ATP assay, and a buffer may be able to chelate the cations thus alleviating the quenching, the potential for significant quenching of ATP light emission due to Cu\(^{2+}\) and Zn\(^{2+}\) in contaminated soils is of concern.

The quenching effect of Ca\(^{2+}\) and Na\(^{+}\) was observed at much lower concentrations than that reported by Aledort et al. (1966) and Karl and LaRock (1975) (Fig. 1). This could be due to the low ATP concentration in the final reaction mixture. Ca\(^{2+}\) was considered to potentially interfere with the luminescence reaction by substitution for Mg\(^{2+}\). Localized alteration in Ca\(^{2+}\) concentration was considered as the mechanism by which fireflies control their light emission and conserve ATP (Aledort et al., 1966). As Ca and Na are abundant in calcareous and saline soils, high concentrations of Ca and Na in the extractants may result in significant underestimation of soil ATP.

4.2. Effects of specific heavy metal cation

The presence of heavy metals in soils at high concentrations not only decrease soil ATP levels (Brookes and McGrath, 1984), but also significantly reduced the light emitted from ATP bioluminescence reaction (Fig. 2). Although the reaction mixture contains EDTA, which is more effective for chelating trivalent or divalent cations than monovalent (Wu Han University, 1979), it could not prevent the quenching to cease the light emission with Ti\(^{3+}\). Hg\(^{2+}\) is known to inhibit the ATP generating steps of photosynthesis (Singh and Singh, 1987), however, the quenching effect occurred at a higher concentration than did Ti\(^{3+}\). The concentration for quenching to occur with Cr\(^{3+}\) was similar to that of Cu\(^{2+}\) and Zn\(^{2+}\) (Fig. 1).

4.3. Effect of added EDTA

Karl and LaRock (1975) showed that the addition of EDTA may enhance light emission in samples of low ATP content where ionic inhibition is extensive. Since significant light depression at higher cation concentrations was observed in this study, increasing the amount of EDTA above that in the commercial ATP assay kit may be necessary for soil ATP assay.

Because there were no cations added to the reaction mixture, the fact that addition of Na-EDTA increased light emission indicates that besides chelating cations reducing light depression, Na-EDTA itself may have an ability to enhance light emission. The large suppression of light emission with further additions of acid-EDTA or Na-EDTA could be attributed to the chelation of Mg\(^{2+}\) (Karl and...
LaRock, 1975). Mg$^{2+}$ is needed in the ATP luminescence reaction as Mg$^{2+}$ reacts with ATP to form a Mg-ATP complex in the bioluminescence reaction, the substrate for luciferase (Webster et al., 1980). Low concentrations of Mg$^{2+}$ would retard the light emission.

The stability of EDTA chelation is in the order of Mg$^{2+} >$ Ba$^{2+} >$ Li$^+ >$ Na$^+$ for the four cations with almost all other cations having a much higher stability constant than that of Mg$^{2+}$ (Wu Han University, 1979). It is expected that Mg$^{2+}$ will be replaced and released to the solution when other free cations co-exist. Often, high concentrations of Mg$^{2+}$ do not depress light emission. Afghan et al. (1977) obtained 91% ATP recovery rate at Mg$^{2+}$ concentrations of 10 mM. Webster and Leach (1980) proposed optimized Mg$^{2+}$ concentration of 5 mM at ATP concentration of 10 ng ml$^{-1}$. The quenching effect observed in this experiment occurring at the concentration of 2.00 mM was because the commercial products contain Na-EDTA as well as the low ATP concentration used in the reaction mixture (1.9 ng ATP ml$^{-1}$).

4.4. Anion effects

The order of quenching effect was similar to that reported by Karl and LaRock (1975), except for a reverse in the order of CO$_3^{2-}$ and PO$_4^{3-}$. This order was observed at much lower concentrations for both ATP and anions than reported. To correspond to the concentration of ATP in blood cells, a concentration of 7 ng ml$^{-1}$ ATP was used in Karl and LaRock’s experiment.

Again, great caution has to be applied in ATP bioluminescence assay in calcareous and saline soils, as those soils may contain high amount of SO$_4^{2-}$, CO$_3^{2-}$ and Cl$^-$. For agricultural soils, presence of the extractable PO$_4^{3-}$ and NO$_3^-$ may enhance light emission. However, in soils receiving freshly applied chemical fertilizers, N and P concentrations could be high enough to quench light emission.

5. Conclusions

This research has revealed that cations and anions at the concentrations that may be encountered in soil ATP extracts affect ATP bioluminescence reactions, when the commercial ATP assay kit is used. Specific heavy metal cations Ti$^{3+}$, Cr$^{3+}$ and Hg$^{2+}$ are effective in quenching light emission. However, enhanced light emission was observed with PO$_4^{3-}$ and NO$_3^-$ at low concentrations.

The tests for further addition of EDTA above that in the assay kit indicated that the addition of Mg-EDTA produce neither enhanced nor depressed light emission in a wide concentration range, therefore, it is superior to acid-EDTA and Na-EDTA.

Due to the potential interferences in analysis of ATP in soil of high cations and anions such as calcareous soils, saline soils, and soils which have received large application of biosolids or fertilizers may not provide accurate ATP estimation. The implications may be extended to state that the presence of cation and anions in soil extractants may require the preparation of ATP standard solutions with similar chemical composition to the soil extract.

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